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Abbreviations: Area under the curve, AUC; Dipeptidyl peptidase IV, DPP IV; Electrospray ionization mass spectrometry, ESI-MS; Gastric inhibitory polypeptide, GIP; glucagon-like peptide-1(7-36)amide, tGLP-1; Trifluoroacetic acid, TFA.

Received 29-03-99 16:49 From-44 1232 320442 To-THE PATENT OFFICE Page 05

Gastric inhibitory polypeptide (GIP) is an important insulin-releasing hormone of the enteroinsular axis which like glucagon-like peptide-1(7-36)amide (tGLP-1) has a functional profile of possible therapeutic value for NIDDM. Both incretin hormones are rapidly inactivated in plasma by the exopeptidase dipeptidyl peptidase IV (DPP IV). The present study has examined the ability of N-terminal modification of human GIP to protect from plasma degradation and enhance insulin-releasing and antihyperglycemic activity. Degradation of GIP by incubation at 37°C with purified DPP IV was clearly evident after 4 h (54% intact). After 12 h, more than 60% of GIP was converted to GIP(3-42) whereas >99% N-terminally modified Tyr^1-glucitol GIP remained intact. Tyr^1-glucitol GIP was similarly resistant to serum degradation. The formation of GIP(3-42) was almost completely abolished by inhibition of plasma DPP IV with diprotin A. Effects of GIP and Tyr^1-glucitol GIP were examined in Wistar rats following i.p. injection of either peptide (10 nmol/kg) together with glucose (18 mmol/kg). Plasma glucose concentrations were significantly lower and insulin concentrations higher following both peptides compared with glucose alone. More importantly, individual glucose values at 15 min and 30 min together with the areas under the curve (AUC) for glucose were significantly lower following administration of Tyr^1-glucitol GIP as compared to GIP (AUC, 255±33 versus 368±8 mmol/l.min, respectively; $P<0.01$). This was associated with a significantly greater and more protracted insulin response following Tyr^1-glucitol GIP than GIP (AUC, 773±41 versus 639±39 ng/ml.min; $P<0.05$). These data demonstrate that Tyr^1-glucitol GIP displays resistance to plasma DPP IV degradation and exhibits enhanced antihyperglycemic activity and insulin-releasing action in vivo.

Received 29-03-99 16:43   From-44 1232 320442   To-THE PATENT OFFICE   Page 06

INTRODUCTION

Gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1(7-36)amide (truncated GLP-1; tGLP-1) are two important insulin-releasing hormones secreted from endocrine cells in the intestinal tract in response to feeding [1,2]. Together with autonomic nerves they play a vital supporting role to the pancreatic islets in the control of blood glucose homeostasis and nutrient metabolism [1,3].

Dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) has been identified as a key enzyme responsible for inactivation of GIP and tGLP-1 in serum [4,5]. DPP IV is completely inhibited in serum by the addition of diprotin A (DPA, 0.1 mmol/l) [4]. This occurs through the rapid removal of the N-terminal dipeptides Tyr^1-Ala^2 and His^7-Ala^8 giving rise to the main metabolites GIP(3-42) and GLP-1(9-36)amide, respectively. These truncated peptides are reported to lack biological activity or to even serve as antagonists at GIP or tGLP-1 receptors [6-9]. The resulting biological half-lives of these incretin hormones in vivo are therefore very short, estimated to be no longer than approximately 5 min, respectively [5,10-12]. In situations of normal glucose regulation and pancreatic B-cell sensitivity, this short duration of action is advantageous in facilitating momentary adjustments to homeostatic control. However, the current goal of a possible therapeutic role of incretin hormones, particularly tGLP-1 in NIDDM therapy is frustrated by a number of factors in addition to finding a convenient route of administration [13]. Most notable of these are rapid peptide degradation and rapid absorption (peak concentrations reached 20 min) and the resulting need for both high dosage and precise timing with meals [13-15]. Recent therapeutic strategies have focussed on precipitated preparations to delay peptide absorption [16] and inhibition of GLP-1 degradation using specific inhibitors of

Received 29-03-99 16:43 From-44 1232 320442 To-THE PATENT OFFICE Page 07

DPP IV [17-19]. A possible therapeutic role is also suggested by the observation that a specific inhibitor of DPP IV, isoleucine thiazolidide, lowered blood glucose and enhanced insulin secretion in glucose-treated diabetic obese Zücker rats presumably by protecting against catabolism of the incretin hormones tGLP-1 and GIP [18].

Numerous studies have indicated that tGLP-1 infusion restores pancreatic B-cell sensitivity, insulin secretory oscillations and improved glycemic control in various groups of patients with IGT or NIDDM [13,15,20-22]. Longer term studies also show significant benefits of tGLP-1 injections in NIDDM and possibly IDDM therapy [20,23,24], providing a major incentive to develop an orally effective or long-acting tGLP-1 analogue [13]. Several attempts have been made to produce structurally modified analogues of tGLP-1 which are resistant to DPP IV degradation [25-27]. A significant extension of serum half-life is observed with His^7-glucitol tGLP-1 and tGLP-1 analogues substituted at position 8 with Gly, Aib, Ser or Thr [25-27]. However, these structural modifications appear to impair receptor binding and insulinotropic activity thereby compromising the part of the benefits of protection from proteolytic degradation [25-28]. Thus in our own recent studies using His^7-glucitol tGLP-1, resistance to DPP IV and serum degradation was accompanied by severe loss of insulin-releasing activity [26,28].

GIP shares not only the same degradation pathway as tGLP-1 but many similar physiological actions, including stimulation of insulin and somatostatin secretion, enhancement of glucose disposal [1]. These actions are viewed as key aspects in the antihyperglycemic properties of tGLP-1 [13], and there is therefore good expectation that GIP may have similar potential in NIDDM therapy. Indeed, compensation by GIP is held to explain the modest disturbances of

glucose homeostasis observed in tGLP-1 knockout mice [29]. Apart from early studies [30], the anti-diabetic potential of GIP has not been explored and tGLP-1 may seem more attractive since it is viewed by some as a more potent insulin secretagogue when infused at 'so called' physiological concentrations estimated by RIA [31].

In a recent study, we have shown that N-terminal glycation of GIP markedly enhances the insulin releasing effect of the peptide on clonal B-cells [32]. If such structural modification also confers DPP IV resistance, the potential attractiveness of this peptide for possible NIDDM therapy would be considerable enhanced. The present study has explored this issue by examining in vitro degradation of Tyr1-glucitol GIP together with evaluation of it's antihyperglycemic and insulin-releasing properties in vivo. The results demonstrate clearly that this novel GIP analogue exhibits a substantial resistance to aminopeptidase degradation and increased glucose lowering activity compared with the native human GIP.

RESEARCH DESIGN AND METHODS

Materials. Human GIP was purchased from the American Peptide Company (Sunnyvale, CA, USA). HPLC grade acetonitrile was obtained from Rathburn (Walkersburn, Scotland). Sequencing grade trifluoroacetic acid (TFA) was obtained from Aldrich (Poole, Dorset, UK). All other chemicals purchased including dextran T-70, activated charcoal, sodium cyanoborohydride and bovine serum albumin fraction V were from Sigma (Poole, Dorset, UK). Diprotin A (DPA) was purchased from Calbiochem-Novabiochem (UK) Ltd. (Beeston, Nottingham, UK) and rat insulin standard for RIA was obtained from Novo

Industria (Copenhagen, Denmark). Reversed-phase Sep-Pak cartridges (C-18) were purchased from Millipore-Waters (Milford, MA, USA). All water used in these experiments was purified using a Milli-Q, Water Purification System (Millipore Corporation, Milford, MA, U.S.A.).

Preparation of Tyr^1-glucitol GIP. Tyr^1-glucitol GIP was prepared and purified by HPLC as described previously [32]. In brief, human GIP was incubated with glucose under reducing conditions in 10 mmol/l sodium phosphate buffer at pH 7.4 for 24 h. The reaction was stopped by addition of 0.5 mol/l acetic acid (30 µl) and the mixture applied to a Vydac (C-18) (4.6 x 250 mm) analytical HPLC column (The Separations Group, Hesperia, CA, USA) and gradient elution conditions were established using aqueous/TFA and acetonitrile/TFA solvents, as described previously. Fractions corresponding to the glycated peaks were pooled, taken to dryness under vacuum using an AES 1000 Speed-Vac concentrator (Life Sciences International, Runcorn, UK) and purified to homogeneity on a Supelcosil (C-8) (4.6 x 150 mm) column (Supelco Inc., Poole Dorset, UK).

Degradation of GIP and Tyr^1-glucitol GIP by DPP IV. HPLC-purified GIP or Tyr^1-glucitol GIP were incubated at 37°C with DPP-IV (5 mU) for various time periods in a reaction mixture made up to 500 µl with 50 mmol/l triethanolamine-HCl, pH 7.8 (final peptide concentration 1 µmol/l) [4]. Enzymatic reactions were terminated after 0, 2, 4 and 12 h by addition of 5 µl of 10% (v/v) TFA/water. Samples were made up to a final volume of 1.0 ml with 0.12% (v/v) TFA and stored at -20°C prior to HPLC analysis.

Degradation of GIP and Tyr^1-glucit 1 GIP by human plasma. Pooled human plasma (20 µl) taken from six healthy fasted male subjects was incubated at 37°C with GIP or Tyr^1-glucitol GIP (10 µg) for 0 and 4 h in a reaction mixture made up to 500 µl, containing 50 mmol/l triethanolamine/HCl buffer pH 7.8. Incubations for 4 h were also performed in the presence of diprotin A (5 mU). The reactions were terminated by addition of 5 µl of TFA and the final volume adjusted to 1.0 ml using 0.1% v/v TFA/water. Samples were centrifuged (13,000*g*, 5 min) and the supernatant applied to a C-18 Sep-Pak cartridge (Millipore-Waters) which was previously primed and washed with 0.1% (v/v) TFA/water. After washing with 20 ml 0.12% TFA/water, bound material was released by elution with 2 ml of 80% (v/v) acetonitrile/water and concentrated using a Speed-Vac concentrator (Runcorn, UK). The volume was adjusted to 1.0 ml with 0.12% (v/v) TFA/water prior to HPLC purification.

HPLC analysis of degraded GIP and Tyr^1-glucitol GIP. Samples were applied to a Vydac C-18 widepore column equilibrated with 0.12% (v/v) TFA/H_2O at a flow rate of 1.0 ml/min. Using 0.1% (v/v) TFA in 70% acetonitrile/H_2O, the concentration of acetonitrile in the eluting solvent was raised from 0% to 31.5% over 15 min, to 38.5% over 30 min and from 38.5% to 70% over 5 min, using linear gradients. The absorbance was monitored at 206 nm and peak areas valuated using a model 2221 LKB integrator. Samples recovered manually were concentrated using a Speed-Vac concentrator.

Electrospray ionization mass spectrometry (ESI-MS). Samples for ESI-MS analysis containing intact and degradation fragments of GIP (from DPP IV and

plasma incubations) as well as Tyr1-glucitol GIP, were further purified by HPLC. Peptides were dissolved (approximately 400 pmol) in 100 µl of water and applied to the LCQ benchtop mass spectrometer (Finnigan MAT, Hemel Hempstead, UK) equipped with a microbore C-18 HPLC column (150 x 2.0 mm, Phenomenex, UK, Ltd., Macclesfield). Samples (30 µl direct loop injection) were injected at a flow rate of 0.2 ml/min, under isocratic conditions 35% (v/v) acetonitrile/water. Mass spectra were obtained from the quadripole ion trap mass analyzer and recorded. Spectra were collected using full ion scan mode over the mass-to-charge (m/z) range 150-2000. The molecular masses of GIP and related structures were determined from ESI-MS profiles using prominent multiple charged ions and the following equation $M_r = iM_i - iM_h$ (where M_r = molecular mass; M_i = m/z ratio; i = number of charges; M_h = mass of a proton).

In vivo biological activity of GIP and Tyr1-glucitol GIP. Effects of GIP and Tyr1-glucitol GIP on plasma glucose and insulin concentrations were examined using 10-12 week old male Wistar rats. The animals were housed individually in an air conditioned room at 22±2°C with a 12 h light/12 h dark cycle. Drinking water and a standard rodent maintenance diet (Trouw Nutrition, Belfast) were supplied ad libitum. Food was withdrawn for an 18 h period prior to intraperitoneal injection of glucose alone (18 mmol/kg body weight) or in combination with either GIP or Tyr1-glucitol GIP (10 nmol/kg). Test solutions were administered in a final volume of 8 ml/kg body weight. Blood samples were collected at 0, 15, 30 and 60 min from the cut tip of the tail of conscious rats into chilled fluoride/heparin microcentrifuge tubes (Sarstedt, Nümbrecht, Germany). Samples were centrifuged using a Beckman microcentrifuge for 30

Received 29-03-99 16:43 From-44 1232 320442 To-THE PATENT OFFICE Page 12

sec at 13,000 *g*. Plasma samples were aliquoted and stored at -20°C prior to glucose and insulin determinations. All animal studies were done in accordance with the Animals (Scientific Procedures) Act 1986.

Analyses. Plasma glucose was assayed by an automated glucose oxidase procedure using a Beckman Glucose Analyzer II [33]. Plasma insulin was determined by dextran charcoal radioimmunoassay as described previously [34]. Incremental areas under plasma glucose and insulin curves (AUC) were calculated using a computer program (CAREA) employing the trapezoidal rule [35] with baseline subtraction. Results are expressed as mean ± SEM and values were compared using the Student's unpaired *t*-test. Groups of data were considered to be significantly different if $P<0.05$.

RESULTS

Degradation of GIP and Tyr¹-glucitol GIP by DPP IV. Fig. 1 illustrates the typical peak profiles obtained from the HPLC separation of the products obtained from the incubation of GIP (left panels) or Tyr¹-glucitol GIP (right panels) with DPP IV for 0, 2, 4 and 12 h. The retention times of GIP and Tyr¹-glucitol GIP at t=0 were 21.93 min and 21.75 min, respectively. Degradation of GIP was evident after 4 h incubation (54% intact), and by 12 h the majority (60%) of intact GIP was converted to the single product with a retention time of 21.61 min. Tyr¹-glucitol GIP remained almost completely intact throughout 2-12 h incubation.

Degradation of GIP and Tyr¹-glucitol GIP by human plasma. Fig. 2 shows a set of typical HPLC profiles of the products obtained from the incubation of GIP or Tyr¹-

glucitol GIP with human plasma for 0 and 4 h. GIP (left panels) with a retention time of 22.06 min was readily metabolised by plasma within 4 h incubation giving rise to the appearance of a major degradation peak with a retention time of 21.74 min. In contrast, the incubation of Tyr1-glucitol GIP under similar conditions (right panels) did not result in the formation of any detectable degradation fragments during this time with only a single peak being observed with a retention time of 21.77 min. Addition of diprotin A, a specific inhibitor of DPP IV, to GIP during the 4 h incubation completely inhibited degradation of the peptide by plasma.

Identification of GIP degradation fragments by ESI-MS. Fig. 3 shows the monoisotopic molecular masses obtained for GIP, (panel A), Tyr1-glucitol GIP (panel B) and the major plasma degradation fragment of GIP (panel C) using ESI-MS. The peptides analyzed were purified from plasma incubations as shown in Fig. 2. The exact molecular mass (M_r) of the peptides were calculated using the equation $M_r = iM_l - iM_h$ as defined in Research Design and Methods section. After spectral averaging was performed, prominent multiple charged species $(M+3H)^{3+}$ and $(M+4H)^{4+}$ were detected from GIP at m/z 1661.6 and 1246.8, corresponding to intact M_r 4981.8 and 4983.2 Da, respectively (Fig. 3A). Similarly, for Tyr1-glucitol GIP ($(M+4H)^{4+}$ and $(M+5H)^{5+}$) were detected at m/z 1287.7 and 1030.3, corresponding to intact molecular masses of M_r 5146.8 and 5146.5 Da, respectively (Fig. 3B). The difference between the observed molecular masses of the quadruply charged GIP and the N-terminally modified GIP species (163.6 Da) indicated that the latter peptide contained a single glucitol adduct corresponding to Tyr1-glucitol GIP. Fig. 3C shows the prominent multiply charged species

Received 29-03-99 16:43 From-44 1232 320442 To-THE PATENT OFFICE Page 14

(M+3H)3+ and (M+4H)4+ detected from the major fragment of GIP at m/z 1583.8 and 1188.1, corresponding to intact M_r 4748.4 and 4748.4 Da, respectively. This corresponds with the theoretical mass of the N-terminally truncated form of the peptide GIP(3-42). This fragment was also the major degradation product of DPP IV incubations (data not shown).

Effects of GIP and Tyr1-glucitol GIP on plasma glucose homeostasis. Fig. 4-5 show the effects of i.p. glucose alone (18 mmol/kg) (control group), and glucose in combination with GIP or Tyr1-glucitol GIP (10 nmol/kg) on plasma glucose and insulin concentrations. Compared with the control group, plasma glucose concentrations and area under the curve (AUC) were significantly lower following administration of either GIP or Tyr1-glucitol GIP (Fig. 4A, B). Furthermore, individual values at 15 and 30 min together with AUC were significantly lower following administration of Tyr1-glucitol GIP as compared to GIP. Consistent with the established insulin-releasing properties of GIP, plasma insulin concentrations of both peptide-treated groups were significantly raised at 15 and 30 min compared with the values after administration of glucose alone (Fig. 5A). The overall insulin responses, estimated as AUC were also significantly greater for the two peptide-treated groups (Fig. 5B). Despite lower prevailing glucose concentrations than the GIP-treated group, plasma insulin response, calculated as AUC, following Tyr1-glucitol GIP was significantly greater than after GIP (Fig. 5B). The significant elevation of plasma insulin at 30 min is of particular note, suggesting that the insulin-releasing action of Tyr1-glucitol GIP is more protracted than GIP even in the face of a diminished glycemic stimulus (Fig. 4A, 5A).

DISCUSSION

The forty-two amino acid GIP is an important incretin hormone released into the circulation from endocrine K-cells of the duodenum and jejunum following ingestion of food [36]. The high degree of structural conservation of GIP among species supports the view that this peptide plays an important role in metabolism [12]. Secretion of GIP is stimulated directly by actively transported nutrients in the gut lumen without a notable input from autonomic nerves [12]. The most important stimulants of GIP release are simple sugars [37] and unsaturated long chain fatty acids [38], with amino acids exerting weaker effects [39]. As with tGLP-1, the insulin-releasing effect of GIP is strictly glucose-dependent [30,40]. This affords protection against hypoglycemia and thereby fulfils one of the most d sirable features of any current or potentially new antidiabetic drug [41].

The present results demonstrate for the first time that Tyr^1-glucitol GIP displays profound resistance to serum and DPP IV degradation. Using ESI-MS the present study showed that native GIP was rapidly cleaved in vitro to a major 4748.4 Da degradation product, corresponding to GIP(3-42) which confirmed previous findings using matrix-assisted laser desorption ionization time-of-flight mass spectrometry [42]. Serum degradation was completely inhibited by diprotin A (Ile-Pro-Ile), a specific competitive inhibitor of DPP IV, confirming this as the main enzyme for GIP inactivation in vivo [4,5]. In contrast, Tyr^1-glucitol GIP remained almost completely intact after incubation with serum or DPP IV for up to 12 h. This indicates that glycation of GIP at the amino-terminal Tyr^1 residue masks the potential cleavage site from DPP IV and prevents removal of the Tyr^1-Ala^2 dipeptide from the N-terminus preventing the formation of GIP(3-42).

Consistent with in vitro protection against DPP IV, administration of Tyr^1-

glucitol GIP significantly enhanced the antihyperglycemic activity and insulin-releasing action of the peptide when administered with glucose to rats. Native GIP enhanced insulin release and r duced the glycemic excursion as observed in many previous studies [12,40]. However, amino-terminal glycation of GIP increased the insulin-releasing and antihyperglycemic actions of the peptide by 62% and 38% respectively, as estimated from AUC measurements. Detailed kinetic analysis is difficult due to necessary limitation of sampling times, but the greater insulin concentrations following Tyr^1-glucitol GIP as opposed to GIP at 30 min post-injection is indicative of longer half-life. The glycemic rise was modest in both peptide-treated groups and glucose concentrations following injection of Tyr^1-glucitol GIP were consistently lower than after GIP. Since the insulinotropic actions of GIP are glucose-dependent [30,40], it is likely that the relative insulin-releasing potency of Tyr^1-glucitol GIP is greatly underestimated in the present in vivo experiments.

In keeping with this interpretation, recent in vitro studies in our laboratory using glucose-responsive clonal B-cells showed that the insulin-releasing potency of Tyr^1-glucitol GIP was several orders of magnitude greater than GIP and that it's effectiveness was more sensitive to change of glucose concentrations within the physiological range [32]. Together with the present in vivo observations, this suggests that N-terminal glycation of GIP confers resistance to DPP IV degradation while enhancing receptor binding and insulin secretory effects on the B-cell. These attributes of Tyr^1-glucitol GIP are fully expressed in vivo where DPP IV resistance impedes degradation of the peptide to GIP(3-42), thereby prolonging the half-life and enhancing effective concentrations of the intact biologically active peptide. It is thus possible that glycated GIP is

Received 29-03-99 16:43 From-44 1232 320442 To-THE PATENT OFFICE Page 17

enhancing insulin secretion in vivo both by enhanced potency at the receptor as well as improving DPP IV resistance. Thus numerous studies have shown that GIP(3-42) and other N-terminally modified fragments, including GIP(4-42), and GIP(17-42) are either weakly effective or inactive in stimulating insulin release [4,43-45]. Furthermore, evidence exists that N-terminal deletions of GIP result in receptor antagonist properties in GIP receptor transfected Chinese hamster kidney cells [9], suggesting that inhibition of GIP catabolism would also reduce the possible feedback antagonism at the receptor level by the truncated GIP(3-42).

In addition to it's insulinotropic actions, a number of other potentially important extrapancreatic actions of GIP may contribute to the enhanced antihyperglycemic activity and other beneficial metabolic effects of Tyr^1-glucitol GIP. These include the stimulation of glucose uptake in adipocytes, increased synthesis of fatty acids and activation of lipoprotein lipase in adipose tissue [46-48]. GIP also promotes plasma triglyceride clearance in response to oral fat loading [49]. In liver, GIP has been shown to enhance insulin-dependent inhibition of glycogenolysis [50]. GIP also reduces both glucagon-stimulated lipolysis in adipose tissue as well as hepatic glucose production [51]. Finally, recent findings indicate that GIP has a potent effect on glucose uptake and metabolism in mouse isolated diaphragm muscle [52]. This latter action may be shared with tGLP-1 [53,54] and both peptides have additional benefits of stimulating somatostatin secretion and slowing down gastric emptying and nutrient absorption [1,55].

In conclusion, this study has demonstrated for the first time that the glycation of GIP at the amino-terminal Tyr^1 residue limits GIP catabolism through impairment of the proteolytic actions of serum peptidases and thus

prolongs its half-life in vivo. This effect is accompanied by enhanced antihyperglycemic activity and raised insulin concentrations in vivo, suggesting that such DPP IV resistant analogues should be explored alongside tGLP-1 as potentially useful therapeutic agents for NIDDM. Tyr^1-glucitol GIP appears to be particularly interesting in this regard since such amino-terminal modification of GIP enhances [32] rather than impairs glucose-dependent insulinotropic potency as was observed recently for tGLP-1 [28].

Acknowledgements

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References

1. Brown JC: Enteroinsular axis. In: *Gut Peptides Biochemistry and Physiology*, Walsh JH, Dockray GJ, Eds. New York, Raven Press, 1994, p. 765-784
2. Herrmann, C; Göke, R.; Richter, G.; Fehmann, H-C; Arnold, R.; Göke, B. Glucagon-like peptide-1 and glucose-dependent insulin releasing polypeptide plasma levels in response to nutrients. *Digestion* 56: 117-126; 1995.
3. Creutzfeldt W: The incretin concept today. *Diabetologia* 16:75-85, 1979
4. Mentlein R, Gallwitz B, Schimdt WE: Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their degradation in human serum. *Eur J Biochem* 214:829-835, 1993
5. Kieffer TJ, McIntosh CHS, Pederson RA: Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide 1 in vitro and in vivo by dipeptidyl peptidase IV. *Endocrinology* 136:3585-3596, 1995
6. Schmidt WE, Siegel EG, Kummel H, Gallwitz B, Creutzfeldt W: Commercially available preparations of porcine glucose-dependent insulinotropic polypeptide (GIP) contain a biologically inactive GIP-fragment and cholecystokinin-33/-39. *Endocrinology* 120:835-837, 1987
7. Gefel D, Hendrick GK, Mojsov S, Habener J, Weir GC: Glucagon-like peptide-1 analogs: effects on insulin secretion and 3',5'-monophosphate formation. *Endocrinology* 126:2164-2168, 1990
8. Grandt D, Sieburg B, Schimiczek M, Becker U, Holtmann G, Layer P, Reeve, JR, Eysselein VE, Goebell H, Müeller M: Is GLP-1(9-36)amide an endogenous antagonist at GLP-1 receptors? (Abstract).*Digestion* 55:302A, 1994

9. Gelling RW, Coy DH, Pederson RA, Wheeler MB, Hinke S, Kwan T, McIntosh CHS: $GIP_{6\text{-}30amide}$ contains the high affinity binding region of GIP and is a potent inhibitor of $GIP_{1\text{-}42}$ action in vitro, *Regul Peptides* 69:151-154, 1997

10. Deacon CF, Nauck MA, ToftNielsen M, Pridal L, Willms B, Holst JJ: Both subcutaneously and intravenously administered glucagon-like peptide-1 are rapidly degraded from the NH2-terminus in type II diabetic patients and in healthy subjects. *Diabetes* 44:1126-1131, 1995

11. Ørskov C, Wettergren A, Holst JJ: The metabolic rate and the biological effects of GLP-1 7-36 amide and GLP-1 7-37 in healthy volunteers are identical. *Diabetes* 42:658-661, 1993

12. Pederson RA: Gastric inhibitory polypeptide. In: *Gut Peptides Biochemistry and Physiology,* Walsh JH, Dockray GJ, Eds. New York, Raven Press, 1994, p. 217-259

13. Byrne MM, Göke B: Lessons from human studies with glucagon-like peptide-1: Potential of the gut hormone for clinical use. In: *The insulinotropic gut hormone glucagon-like peptide-1,* Fehmann HC, Göke B, Eds. Basel, Karger, 1997, p. 219-233

14. Ritzel R, Ørskov C, Holst JJ, Nauck MA: Pharmacokinetic, insulinotropic, and glucagonostatic properties of GLP-1(7-36)amide after subcutaneous injection in healthy volunteers. Dose-response relationships. *Diabetologia* 38:720-725, 1995

15. Nathan DM, Schreiber E, Fogel H, Mojsov S, Habener JF: Insulinotropic action of glucagon-like peptide-1-(7-37) in diabetic and non-diabetic patients. *Diabetes Care* 15:270-276, 1992

16. Rose CA, Kim Y: Precipation of insulinotropin in the presence of protamine: effect of phenol and zinc on the isophane ratio and the insulinotropinin the supernatant. *Pharmacol Res* 12:1284-1288, 1995

17. Deacon CF, Hughes TE, Holst JJ: Dipeptidyl peptidase IV inhibition potentiates the insulinotropic effect of glucagon-like peptide-1 in the anaesthesised pig. *Diabetes* 47:764-769, 1998

18. Pederson RA, White HA, Schlenzig D, Pauly RP, McIntosh CHS, Demuth H-U: Improved glucose tolerance in Zucker fatty rats by oral administration of the dipeptidyl peptidase IV inhibitor isoleucine thiazolidide. *Diabetes* 47:1253-1258, 1998

19. Holst JJ, Deacon CF: Inhibition of the activity of dipeptidyl-peptidase IV as a treatment for Type 2 diabetes. *Diabetes* 47:1663-1670, 1998

20. Gutniak M, Ørskov C, Holst JJ, Ahrén B, Efendic S: Antidiabetogenic effect of glucagon-like peptide-1 (7-36) amide in normal subjects and patients with diabetes mellitus. *N Engl J Med* 326:1326-1322, 1992

21. Nauck MA, Kleine N, Ørskov C, Holst JJ, Willims B, Creutzfeldt W: Normalization of fasting hyperglycaemia by exogenous glucagon-like peptide-1(7-36) in type 2 (non-insulin-dependent) diabetic patients. *Diabetologia* 36:741-744, 1993

22. Byrne MM, Gliem K, Wank U, Arnold R, Katschinski M, Polonsky KS, Göke G: Glucagon-like peptide-1 improves the ability of the β-cell to sense and respond to glucose in subjects with impaired glucose tolerance. *Diabetes* 47:1259-1265, 1998

Received 29-03-99 16:43 From-44 1232 320442 To-THE PATENT OFFICE Page 22

29. Pederson RA, Satkunarajah M, McIntosh CHS, Scrocchi LA, Flamez D, Schuit F, Drucker DJ, Wheeler MB: Enhanced glucose-dependent insulinotropic polypeptide secretion and insulinotropic action in glucagon-like peptide 1 receptor -/- mice. *Diabetes* 47:1046-1052, 1998

30. Elahi D, Andersen DK, Brown JC, Debas H, Hershcopf RJ, Raizes GS, Tobin JD, Andres R: Pancreatic α and β-cell responses to GIP infusion in normal man. *Am J Physiol* 237:185-191, 1979

31. Marks V, Morgan LM: Intra-islet interactions and the enteroinsular axis in insulin secretion. In: *Frontiers of Insulin Secretion and Pancreatic B-cell Research*, Flatt PR, Lenzen S, Eds. London, Smith-Gordon, 1994, p. 319-324

32. O'Harte FPM, Abdel-Wahab YHA, Conlon JM, Flatt PR: Amino terminal glycation of gastric inhibitory polypeptide enhances its insulinotropic action on clonal pancreatic B-cells. *Biochim Biphys Acta* 1425:319-327, 1998

33. Stevens VJ: Determination of glucose by automatic analyser. *Clin Chem* 32:9199-9201, 1971

34. Flatt PR, Bailey CJ: Abnormal plasma glucose and insulin responses in heterozygous lean (ob/+) mice. *Diabetologia* 20:573-577, 1981

35. Burington RS: *Handbook of Mathematical Tables and Formulas*. Burington RS, Ed. New York, McGraw-Hill, 1973

36. Buchan AMJ, Polak JM, Capella C, Solcia E, Pearse AGE: Electron immunocytochemical for the K cell localization of gastric inhibitory polypeptide (GIP) in man. *Histochemistry* 546:37-44, 1978

37. Sykes S, Morgan LM, English J, Marks V: Evidence for preferential stimulation of gastric inhibitory polypeptide secretion in the rat by actively transported carbohydrates and their analogues, *J Endocrinol* 85:201-207, 1980

38. Kwasowski P, Flatt PR, Bailey CJ, Marks V: Effect of fatty acid chain length and saturation on gastric inhibitory peptide release in obese hyperglycaemic (ob/ob) mice. *Biosci Rep* 5:701-705, 1985

39. Flatt PR, Kwasowski P, Howland RJ, Bailey CJ: Gastric inhibitory polypeptide and insulin responses to orally administered amino acids in genetically obese hyperglycaemic (ob/ob) mice. *J Nutr* 121:1123-1128, 1991

40. Pederson RA, Brown JC: The insulinotropic action of gastric inhibitory polypeptide in the perfused isolated rat pancreas. *Endocrinology* 99:780-785, 1976

41. Bailey CJ, Flatt PR: Development of antidiabetic drugs. In: *Drugs, Diet and Disease, Vol 2, Mechanistic Approaches to Diabetes*, Ioannides C, Flatt PR, Eds. London, Ellis Horwood, 1995, p. 279-326

42. Pauly RP, Rosche F, Wermann M, McIntosh CHS, Pederson RA, Demuth HU: Investigation of glucose-dependent insulinotropic polypeptide-(1-42) and glucagon-like peptide-1-(7-36) degradation in vitro by dipeptidylpeptidase IV using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. *J Biol Chem* 271:23222-23229, 1996

43. Maletti M, Carlquist M, Portha B, Kergoat M, Mutt V, Rosselin G: Structural requirements for gastric inhibitory polypeptide (GIP) receptor binding and stimulation of insulin release, *Peptides* 7:75-78, 1986

44. Oektedalen O, Opstad PK, Jorde R: Increased plasma response of gastric inhibitory polypeptide to oral glucose and a liquid meal after prolonged starvation in healthy man. *Digestion* 26:114-123, 1983

45. Moody AJ, Damm Jorgensen K, Thim L: Structure-function relationships in porcine GIP (Abstract).*Diabetologia* 21:306, 1981

46. Eckel RH, Fujimoto WJ, Brunzell JD: Gastric inhibitory polypeptide enhances lipoprotein lipase activity in cultured pre-adipocytes. *Diabetes* 28:1141-1142, 1978

47. Morgan LM: The metabolic role of GIP: Physiology and pathology. *Biochem Soc Trans* 24:585-591, 1996

48. Oben J, Morgan L, Fletcher J, Marks V: Effect of the entero-pancreatic hormones gastric inhibitory polypeptide and glucagon-like peptide-1(7-36)amide on fatty acid synthesis in explants of rat adipose tissue. *J Endocrinol* 130:267-272, 1991

49. Ebert R, Nuack M, Creutzfeldt W: Effects of exogenous and endogenous gastric inhibitory polypeptide on plasma triglyceride responses in rats. *Horm Metab Res* 23:517-521, 1991

50. Elahi D, Meneilly GS, Minaker KL, Rowe JW, Andersen DK: Regulation of hepatic glucose production by gastric inhibitory polypeptide in man. In: *Proceedings of Sixth International Conference on Gastrointestinal Hormones*, Vancouver, BC, p18 National Research Council of Canadian Research Journals, Ottawa, 1986

51. Hartmann H, Ebert R & Creutzfeldt W: Insulin-dependent inhibition of hepatic glycogenolysis by gastric inhibitory polypeptide (GIP) in perfused rat liver. *Diabetologia* 29:112-114, 1986

52. O'Harte FPM, Gray AM, Flatt PR: Gastric inhibitory polypeptide and effects of glycation on glucose transport and metabolism in isolated mouse abdominal muscle. *J Endocrinol* 156:237-243, 1998

53. Villanueva-Peñacarrillo, ML, Alcántara AI, Clemente F, Delgado E, Valverde I: Potent glycogenic effect of GLP-1(7-36)amide in rat skeletal muscle. *Diabetologia* 37:1163-1166, 1994

54. O'Harte FPM, Gray AM, Abdel-Wahab YHA & Flatt PR: Effects of non-glycated and glycated glucagon-like peptide-1(7-36) amide on glucose metabolism in isolated mouse abdominal muscle. *Peptides* 18:1327-1333, 1997

55. Wahren J, Efendic S, Luft R, Hagenfeldt L, Bjorkman O, Felig P: Influence of somatostatin on sphlachnic glucose metabolism in postabsorptive and 60-hour fasted humans. *J Clin Invest* 59:299-307, 1977

Legends t Figures

Fig. 1. Degradation of GIP and Tyr[1]-glucitol GIP by DPP IV. Representative HPLC profiles obtained after incubation of GIP (left panels) or Tyr[1]-glucitol GIP (right panels) with DPP IV for 0, 2, 4 and 12 h. Incubations of GIP and Tyr[1]-glucitol GIP exposed to DPP IV were separated on a Vydac C-18 column using linear gradients 0% to 31.5% acetonitrile over 15 min, to 38.5% over 30 min and from 38.5% to 70% acetonitrile over 5 min. Left hand panels show HPLC profiles of intact GIP (retention time 21.93 min) and GIP(3-42) (retention time 21.61 min). Right hand panels show HPLC profiles obtained for Tyr[1]-glucitol GIP (retention time 21.75 min). HPLC peaks corresponding to intact GIP, GIP(3-42) and Tyr[1]-glucitol GIP are indicated.

Fig. 2. Degradation of GIP and Tyr[1]-glucitol GIP by human plasma. Representative HPLC profiles obtained after incubation of GIP (left panels) and Tyr[1]-glucitol GIP (right panels) with human plasma for 0 and 4 h and for 4 h in the presence of 5 mU of diprotin A (DPA). GIP and Tyr[1]-glucitol GIP incubations were separated with a Vydac C-18 column using linear gradients 0% to 31.5% acetonitrile over 15 min, to 38.5% over 30 min and from 38.5% to 70% acetonitrile over 5 min. Peaks corresponding with intact GIP, GIP(3-42) and Tyr[1]-glucitol GIP are indicated. A major peak corresponding to the specific DPP IV inhibitor tripeptide DPA appears in the bottom panels with retention time 16.29 min.

Fig. 3. Electrospray ionization mass spectrometry of GIP, Tyr1-glucitol GIP and the major degradation fragment GIP(3-42). Samples containing (A) GIP, (B) Tyr1-glucitol GIP and (C) the major degradation fragment of GIP (GIP(3-42)) isolated from plasma incubations (Fig. 2). Peptides were dissolved (approximately 400 pmol) in 100µl of water and applied to the LC/MS equipped with a microbore C-18 HPLC column. Samples (30 µl direct loop injection) were applied at a flow rate of 0.2 ml/min, under isocratic conditions 35% acetonitrile/water. Mass spectra were recorded using a quadripole ion trap mass analyzer. Spectra were collected using full ion scan mode over the mass-to-charge (m/z) range 150-2000. The molecular masses (M_r) of GIP and related structures were determined from ESI-MS profiles using prominent multiple charged ions and the following equation $M_r = iM_i - iM_h$ (see Research Design and Methods section).

Fig. 4. Effects of GIP and glycated GIP on plasma glucose homeostasis. (A) Plasma glucose concentrations after i.p. glucose alone (18 mmol/kg) (control group), or glucose in combination with either GIP or Tyr1-glucitol GIP (10 nmol/kg). The time of injection is indicated by the arrow (0 min). (B) Plasma glucose AUC values for 0-60 min post injection. Values are mean ± SEM for six rats. $^{**}P<0.01$, $^{***}P<0.001$ compared with GIP and Tyr1-glucitol GIP; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ compared with non-glycated GIP.

Fig. 5. Effects of GIP on plasma insulin respons s. (A) Plasma insulin concentrations after i.p. glucose alone (18 mmol/kg) (control group), or glucose in combination with either with GIP or glycated GIP (10 nmol/kg). The time of injection is indicated by the arrow. **(B)** Plasma insulin AUC values were calculated for each of the 3 groups up to 90 min post injection. The time of injection is indicated by the arrow (0 min). Plasma insulin AUC values for 0-60 min post injection. Values are mean ± SEM for six rats. *P<0.05, **P<0.01 ***P<0.001 compared with GIP and Tyr^{1}-glucitol GIP; †P<0.05, ††P<0.01 compared with non-glycated GIP.

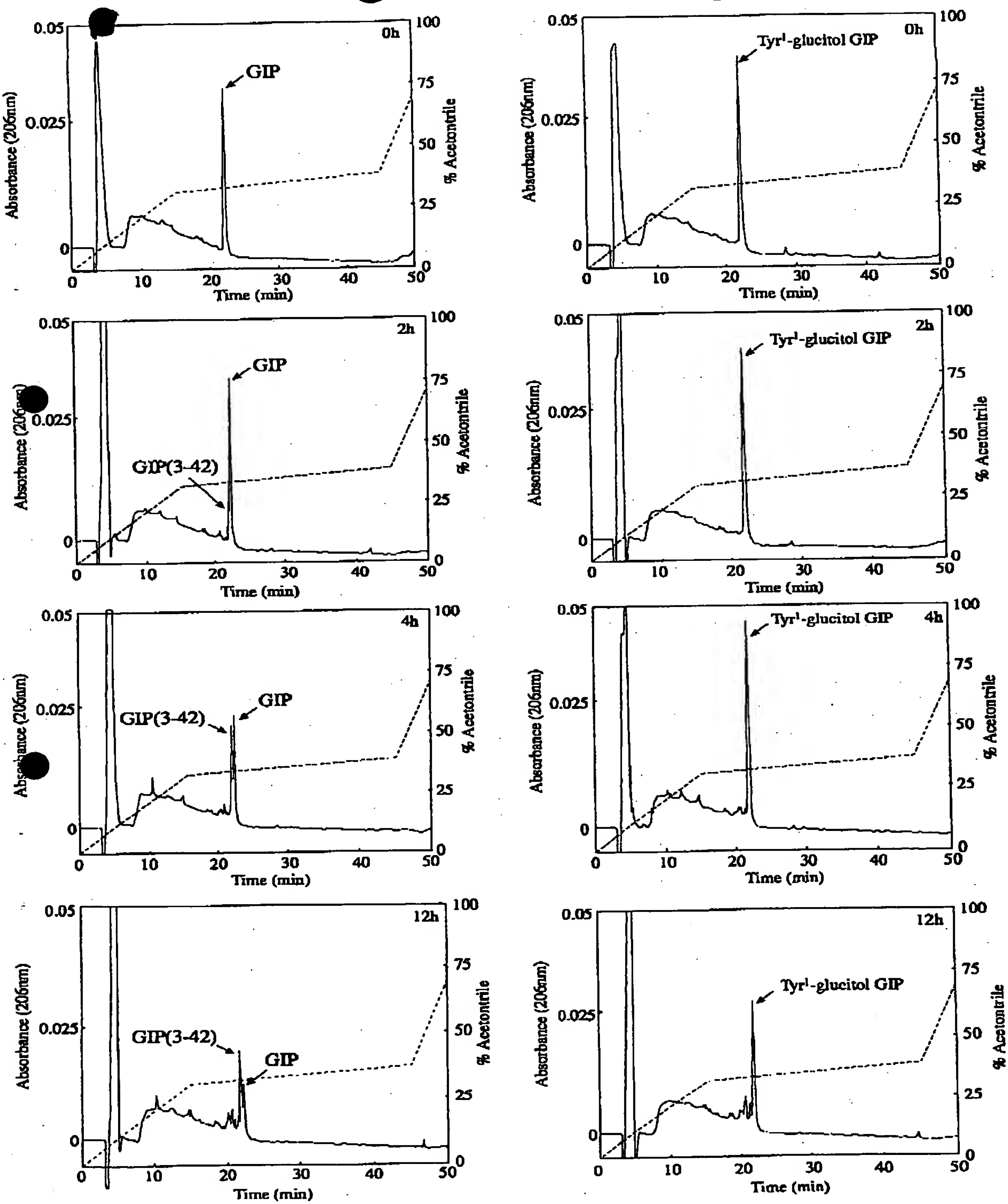
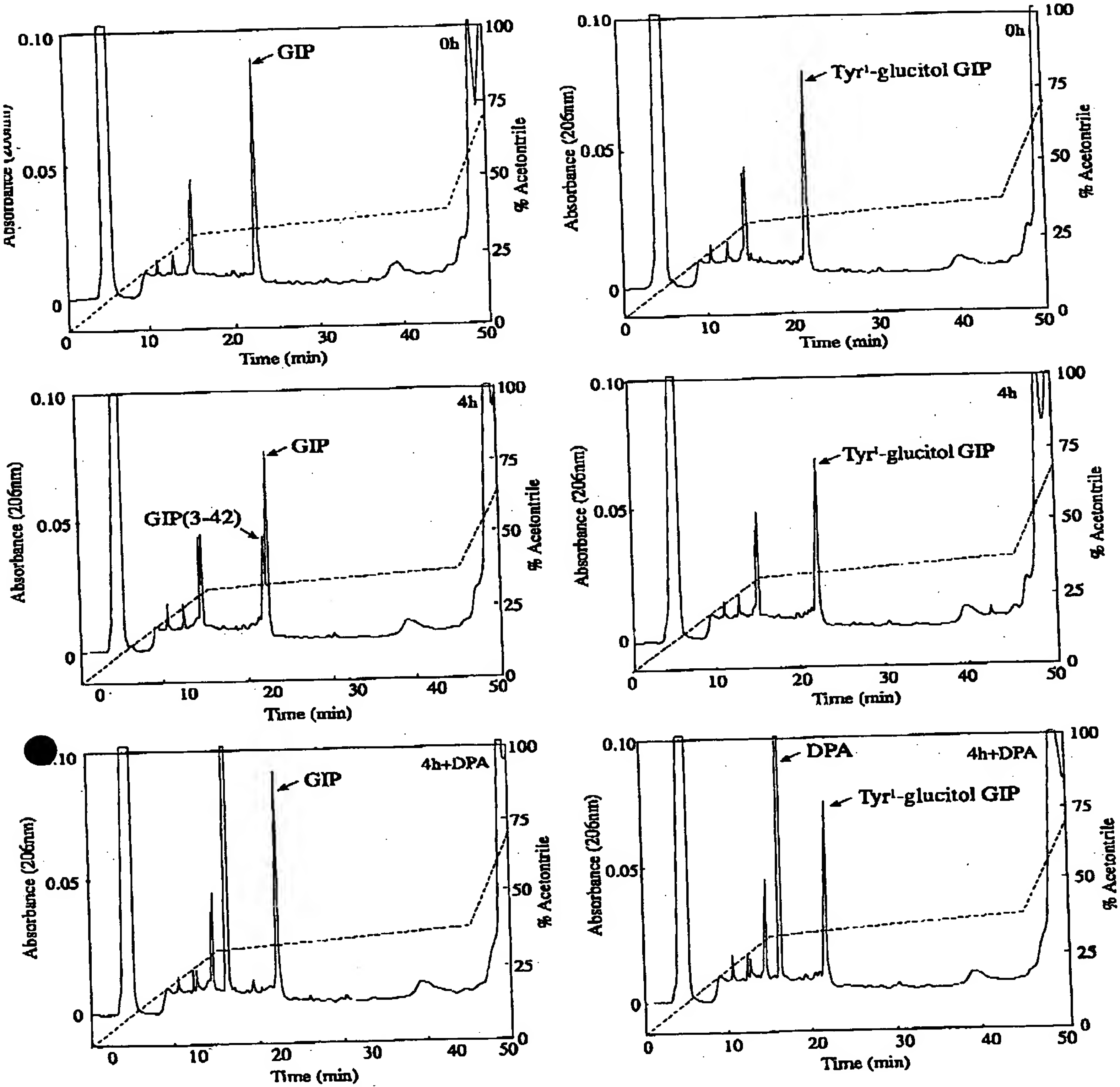

0h
2h
4h
12h
GIP
GIP(3-42)
Tyr¹-glucitol GIP
Absorbance (206nm)
% Acetonitrile
Time (min)

Fig 2


0.10
0.05
0
0h
GIP
Tyr¹-glucitol GIP
4h
GIP(3-42)
4h+DPA
DPA
Absorbance (206nm)
% Acetontrile
100
75
50
25
0
10
20
30
40
50
Time (min)

Fg3

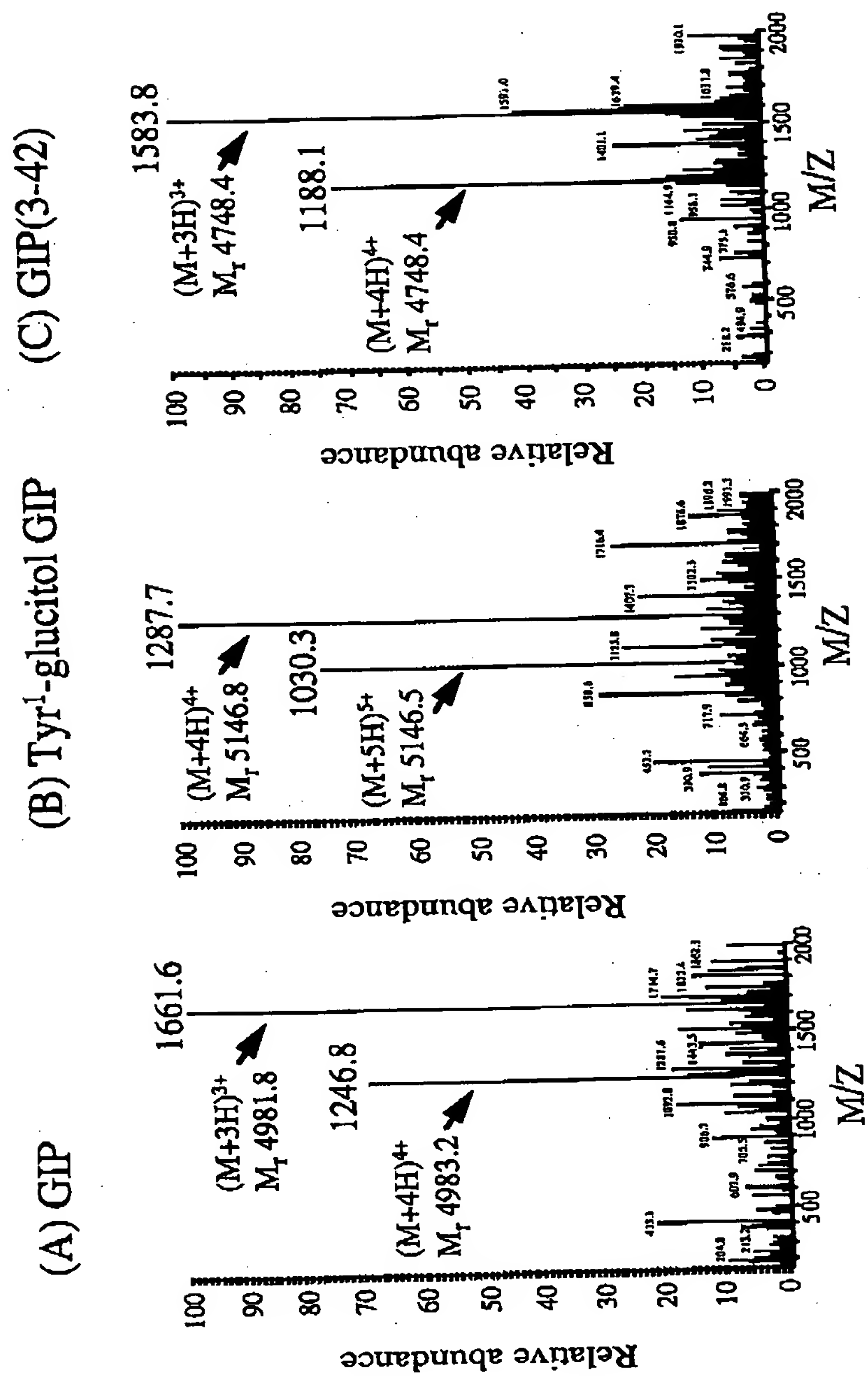

(A) GIP
(B) Tyr1-glucitol GIP
(C) GIP(3-42)
1661.6
(M+3H)3+
Mr 4981.8
1246.8
(M+4H)4+
Mr 4983.2
1287.7
(M+4H)4+
Mr 5146.8
1030.3
(M+5H)5+
Mr 5146.5
1583.8
(M+3H)3+
Mr 4748.4
1188.1
(M+4H)4+
Mr 4748.4
Relative abundance
M/Z

Fig 4

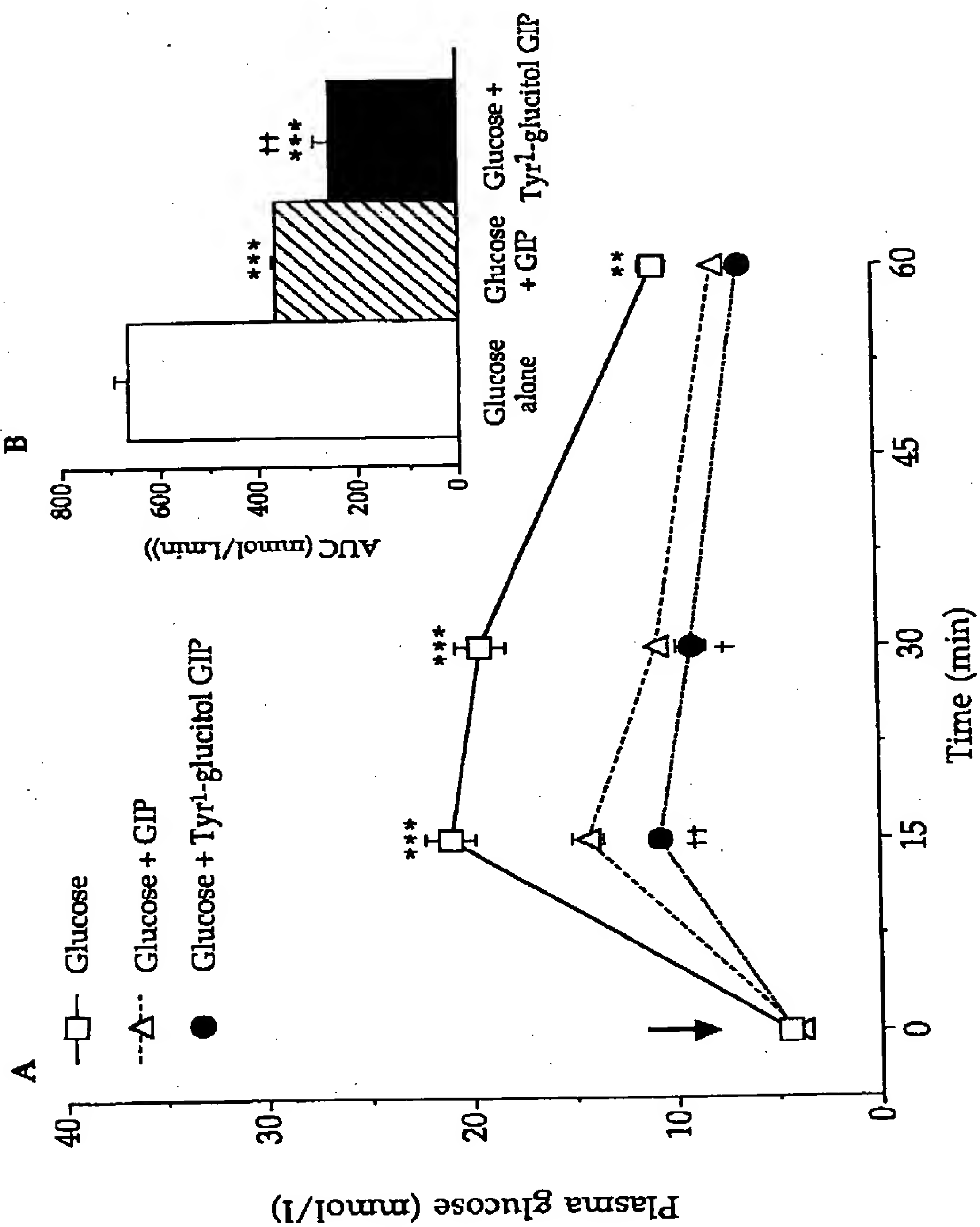
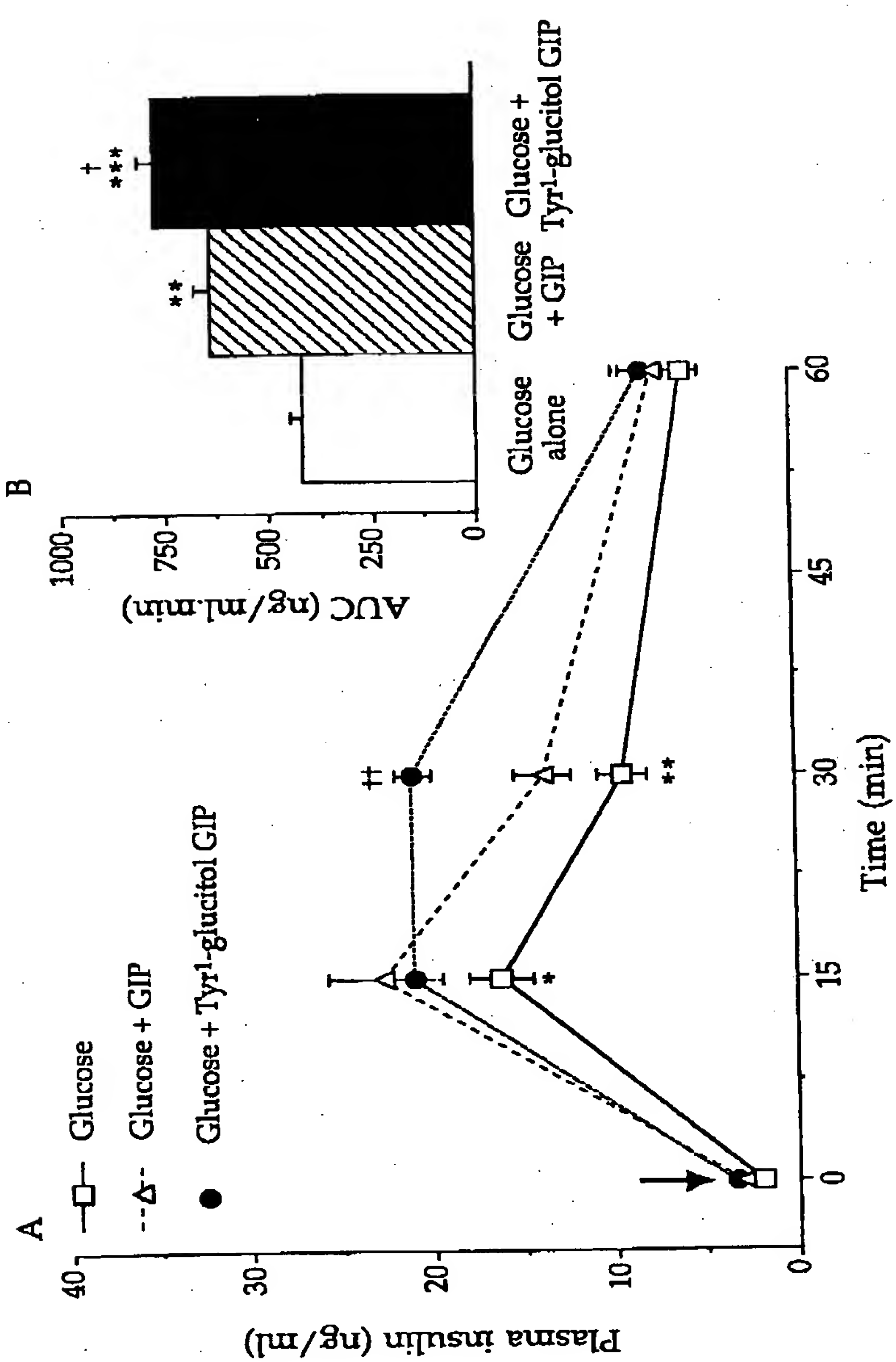

A
Glucose
Glucose + GIP
Glucose + Tyr1-glucitol GIP
Plasma glucose (mmol/l)
40
30
20
10
0
Time (min)
0
15
30
45
60
***
**
++
+
B
AUC (mmol/l.min)
800
600
400
200
0
Glucose alone
Glucose + GIP
Glucose + Tyr1-glucitol GIP

Fig 5


A
Glucose
Glucose + GIP
Glucose + Tyr1-glucitol GIP
Plasma insulin (ng/ml)
40
30
20
10
0
0
15
30
45
60
Time (min)
B
AUC (ng/ml.min)
1000
750
500
250
0
Glucose alone
Glucose + GIP
Glucose + Tyr1-glucitol GIP

PCT/GB00/01089
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